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Nicotinic acetylcholine receptors containing α6 subunits contribute to alcohol reward-related behaviours

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Evidence is emerging that neuronal nicotinic acetylcholine receptors (nAChRs) in the mesolimbic dopamine (DA) system are involved in mediating the reinforcing effects of alcohol. Midbrain DA neurons express high levels of α6 subunit-containing nAChRs that modulate DA transmission, implicating their involvement in reward-related behaviours. This study assessed the role of α6-containing nAChRs in modulating alcohol reward using transgenic mice expressing mutant, hypersensitive α6 nAChR subunits (α6L9′S mice). α6L9′S mice and littermate controls were tested in three well-established models of alcohol reward: 24-h two-bottle choice drinking, drinking in the dark (DID), and conditioned place preference (CPP). Confocal microscopy and patch-clamp electrophysiology were used to show the localization and function of hypersensitive α6 subunit-containing nAChRs. Results indicate that female α6L9′S mice showed significantly higher alcohol intake at low concentrations of alcohol (3% and 6%) in the two-bottle choice procedure. Both male and female α6L9′S mice drank significantly more in the DID procedure and displayed an alcohol-induced place preference using a low dose of alcohol (0.5 g/kg) that was ineffective in littermate controls. Confocal microscopy showed that α6 subunit-containing nAChRs are selectively expressed on ventral tegmental area (VTA) DAergic, but not GABAergic neurons. Patch-clamp electrophysiology showed that VTA DA neurons of α6L9′S mice are hypersensitive to ACh. Collectively, these results suggest that α6L9′S mice are more sensitive to the rewarding effects of alcohol, and suggest that VTA α6 subunit-containing nAChRs modulate alcohol reward. Thus, α6 subunit-containing nAChRs may be a promising therapeutic target for treatment of alcohol use disorders.

Keywords: Acetylcholine, alcohol, conditioned place preference, confocal microscopy, dopamine, drinking, mice, nicotinic receptor, nucleus accumbens, patch-clamp electrophysiology, reward, transgenic, ventral tegmental area, α6 subunit

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Alcohol and nicotine are often used together (Grant *et al*. 2004), possibly because the two drugs interact to influence the rewarding effects of one or both substances (Little 2000). Nicotine exerts its effects on nicotinic acetylcholine receptors (nAChRs), which are widely expressed in the central nervous system. Nicotinic acetylcholine receptors can modulate the excitability of neurons, ultimately affecting the release of neurotransmitters, including acetylcholine (ACh), γ-Aminobutyric acid (GABA) (Mcclure-Begley *et al*. 2009), glutamate (Garduno *et al*. 2012; Mansvelder & Mcgehee 2000), norepinephrine (Azam & Mcintosh 2006) and serotonin (Gotti *et al*. 2006). Importantly, presynaptic nAChRs located in the mesolimbic reward pathway can strongly modulate dopamine (DA) release (Drenan *et al*. 2010, 2008; Grady *et al*. 2002; Jackson *et al*. 2009; Salminen *et al*. 2007).

Alcohol elevates DA levels in the nucleus accumbens (Söderpalm *et al*. 2009), a brain area involved in the reinforcing properties of alcohol (reviewed by Wise 1998). Evidence suggests that nAChRs in the ventral tegmental area (VTA) may underlie elevated alcohol-induced accumbal DA levels. Ericson and colleagues (2008) showed that when the non-selective nAChR antagonist mecamylamine was locally administered to the anterior VTA, it blocked alcohol-induced increases in accumbal DA levels, suggesting that nAChRs in the VTA may be involved in modulating alcohol reward. Mecamylamine also reduced alcohol drinking in rodents (Blomqvist *et al*. 1996; Ericson *et al*. 1998; Hendrickson *et al*. 2010) and humans (Chi & De Wit 2003; Young *et al*. 2005). The FDA-approved smoking cessation aid varenicline, a partial agonist at α4β2* and α6β2* nAChRs (* indicates that other subunits may be present in the pentameric receptor) (Mihalak *et al*. 2006), reduced alcohol intake in rodents (e.g. Hendrickson *et al*. 2010) and in humans (Mckee *et al*. 2009). However, because both mecamylamine and varenicline are not selective antagonists, it is not clear which nAChR subtypes are involved in alcohol drinking behaviour.

The mesolimbic DA system, consisting in part of DA neurons in the VTA and their projections to nucleus accumbens, is crucial to alcohol reward (e.g. Brodie *et al*.

1999; Gessa *et al*. 1985). α6* nAChRs are enriched in the mesolimbic reward pathway, and previous studies support the claim that α6* nAChRs, unlike α4 (non-α6)* nAChRs, are selectively expressed in DA neurons but not in local GABA neurons that inhibit DA cells. Recently, Drenan and colleagues (2008) created mice with mutated α6 nAChR subunits (α6L9′S mice) in order to selectively amplify the downstream effects of activating α6* nAChRs. In α6L9′S mice, α6* nAChRs are markedly hypersensitive to nicotine, showing greater nicotine-stimulated DA release in synaptosome experiments and larger inward currents in response to local application of nicotine to patch-clamped VTA DA neurons in brain slices (Drenan *et al*. 2008). These results provide evidence that α6* nAChRs located on VTA DAergic neurons modulate DA transmission.

Indirect evidence suggests that α6* nAChRs may play an important role in modulating alcohol reward-related behaviours. α-Conotoxin MII, an antagonist at both α3β2* and α6* nAChRs, reduced both voluntary drinking (Larsson & Engel 2004) and operant responding (Löf *et al*. 2007) for alcohol. However, because α-conotoxin MII is not perfectly selective for α6* vs. α3* nAChRs, complementary approaches are necessary to confirm the role of specific nAChR subunits in modulating behaviour. Mutant mice with hypersensitive α6* nAChRs provide an excellent opportunity to evaluate the specific role of the α6 subunit in alcohol reward.

The purpose of this study was to assess the involvement of α6* nAChRs in modulating alcohol reward by comparing α6L9′S mice to littermate controls in three well-established models of alcohol reward: 24-h two-bottle choice drinking, drinking in the dark (DID) and alcohol-induced conditioned place preference (CPP). Confocal microscopy and patch-clamp electrophysiology were used to show the localization and function of hypersensitive α6* nAChRs.

Materials and methods

Subjects

α6L9′S bacterial artificial chromosome (BAC) transgenic mice were constructed as previously described (Drenan *et al*. 2008). Briefly, an ectopic BAC containing a mutant, L9′S allele of the mouse α6 nAChR subunit gene was introduced into fertilized FVB/N embryos and implanted into pseudopregnant Swiss-Webster surrogates. The insertion site within the mouse genome is unknown. Founder animals were back-crossed to C57BL/6 12–15 times prior to these studies. Although >90% of the genome is expected to be C57BL/6 following this many rounds of back-crossing, elimination of FVB/N allelic DNA immediately adjacent to the BAC insertion site is not likely. α-Conotoxin MII binding experiments showed that α6* nAChRs are faithfully expressed in their correct anatomical locations and are not overexpressed in α6L9′S mice (Drenan *et al*. 2010, 2008). Both male and female mice were used in the current experiments with ages ranging from 64 to 193 days old at the time of experimental procedures. Mice were group housed (unless otherwise indicated) and had free access to food (Rodent Lab Diet 5001, Purina Mills Inc., St. Louis, MO, USA) and water in the home-cage. Ambient temperature in the colony and experimental rooms was maintained at 21 ± 2°C. Efforts were made to minimize any pain and discomfort to the mice. All experiments were conducted in accordance with the guidelines for care and use of animals provided by the National Institutes of Health (NIH), and all procedures were approved by the Purdue University Institutional Animal Care and Use Committee.

Drugs

Alcohol (95% v/v) was diluted with tap water (drinking studies) or saline (CPP studies) to obtain the desired concentrations (3%, 6%, 10% and 20% v/v). For the place conditioning study, alcohol was administered intraperitoneally (i.p.) in doses of 0.5 and 2.0 g/kg of body weight with injection volumes of 3.15 and 12.6 ml/kg (20% v/v), respectively.

Place conditioning apparatus

The place conditioning apparatus has been utilized in prior place conditioning studies with mice (e.g. Powers *et al*. 2010). The apparatus consisted of 12 identical Plexiglas boxes (43.2 × 1.6 × 25.4 cm) enclosed in separate ventilated sound- and light-attenuated chambers (76.2 × 50.8 × 20.3 cm). The floor of each box consisted of interchangeable halves with distinct floor textures. One floor texture (the Grid floor) consisted of 4 mm steel rods mounted 3.5 mm apart and the other floor texture (the Hole floor) was made up of perforated 16 gauge stainless steel with 6.4 mm holes on 9.5 mm staggered centres. Locomotor activity and side position (left or right) for each mouse was continuously monitored in each box with an open field activity frame (SmartFrame Low Density, Lafayette Instrument Co, Lafayette, IN, USA) that contained infrared photobeams along the length and width of each frame (internal frame dimensions: 24.1 × 45.7 cm). Ventilation fans in each box masked outside noise.

Study procedures

Twenty-four-hour continuous-access alcohol drinking

Mice [*n* = 35; 17 α6L9′S (9 male/8 female) and 18 non-Tg (10 male/8 female)] were singly housed for 7 days with access to two water-filled tubes (25 ml fitted with metal drinking spouts) in order to habituate them to the experimental environment. On the eighth day, one of the water tubes was replaced with an alcohol tube for each mouse. Alcohol concentration was increased every 4 days: days 1–4 (3%), days 5–8 (6%), days 9–12 (10%) and days 13–16 (20%). Every 2 days, mice were weighed, drinking solutions were replaced and bottle position was alternated to avoid the development of a side preference (Kamens *et al*. 2010). Evaporation/leakage was estimated from tubes placed on empty cages, and mean volume lost was subtracted from individual drinking values before analysis.

Drinking in the dark

The DID experiment was conducted as previously described (Rhodes *et al*. 2005). The same mice used in the 24-h continuous-access alcohol drinking study remained singly housed for 33 days with normal water bottles. Mice then received one water-filled drinking tube on the home cage for 7 days. On days 8 through 11, body weights were measured and the water tube for each mouse was replaced with a 20% v/v alcohol tube 2 h after lights off (lights off at 0945 h). Mice had access to alcohol for 2 h. Amount of alcohol consumed was recorded daily after each 2-h session.

Alcohol-induced CPP

The place conditioning procedure consisted of one pretest, eight conditioning sessions and one post-test. All phases of the experiment were conducted on consecutive days except that a 48-h break separated the first four and the second four conditioning sessions. Experimental groups were counterbalanced by genotype, sex, order of exposure to the conditioning stimulus (CS), box enclosure and floor position (i.e. left vs. right side of the box). All animals were transferred on a cart to the procedure room 30 min before the start of experimental procedures each day.

Pretest (Day 1). Initial unconditioned preference for each CS (Grid vs. Hole) was assessed 24-h prior to the start of the first conditioning trial. Subjects were injected (i.p.) with saline (at a volume equal to a 0.5 or 2.0 g/kg dose of alcohol, depending on the experiment) and placed in the testing box with free access to both floor types for 60 min.

Conditioning sessions (Days 2–11). Mice underwent a differential place conditioning procedure in which they were randomly assigned to one of two conditioning subgroups within each experimental subgroup. Each conditioning subgroup received 5 min of exposure to either a Grid or Hole floor paired with alcohol treatment (4 CS+ conditioning sessions) and the other floor type paired with saline (4 CS− conditioning sessions) on alternating days, for a total of eight conditioning sessions. The floors and inside of the box were wiped with a damp sponge between each subject.

Separate experiments were run for each dose of alcohol (0.5 and 2.0 g/kg), but all procedures were identical. A total of 32 mice [17 α6L9′S (8 male/9 female) and 15 non-Tg (4 male/11 female)] were used for the 0.5 g/kg experiment, and 28 mice [17 α6L9′S (9 male/8 female) and 11 non-Tg (3 male/8 female)] were used for the 2.0 g/kg experiment. The low dose of alcohol (0.5 g/kg) was chosen because we hypothesized that α6L9′S mice would be more sensitive to alcohol reward, and a 0.5 g/kg dose is below the threshold for producing alcohol-induced CPP in C57BL/6 mice (e.g. Cunningham *et al*. 1992; Wrobel 2011). The 2.0 g/kg dose was used as a positive control.

Post-test (Day 12). Mice were tested for CPP 24-h after their final conditioning session. As in the pretest, mice had free access to both floors in the same position for 60 min. Mice received an injection of saline (equal volume to a 0.5 or 2.0 g/kg dose of alcohol) directly before being placed in the apparatus to match cues during conditioning.

Brain slice preparation for electrophysiology

Brain slices were prepared as previously described (Engle *et al*. 2012). α6L9′S and non-Tg mice were genotyped at 21–28 days after birth. Mice were anesthetized with sodium pentobarbital (100 mg/kg; i.p.) followed by cardiac perfusion with oxygenated (95% O_2/5% CO_2), 4°C *N*-methyl-D-glucamine (NMDG)-recovery solution containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na^+ ascorbate, 2 thiourea, 3 Na^+ pyruvate, 10 $MgSO_4{\cdot}7H_2O$, 0.5 $CaCl_2{\cdot}2H_2O$ (300–310 mOsm, pH 7.3–7.4). Brains were removed and retained in 4°C NMDG-recovery solution for 1 min. Coronal slices (250 μm) were cut with a microslicer (DTK-Zero 1; Ted Pella, Redding, CA, USA). Brain slices recovered for 12 min at 33°C in oxygenated NMDG-recovery solution, after which they were held until recording in HEPES holding solution containing (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na^+ ascorbate, 2 thiourea, 3 Na^+ pyruvate, 2 $MgSO_4{\cdot}7H_2O$, 2 $CaCl_2{\cdot}2H_2O$ (300–310 mOsm, pH 7.3–7.4). Coordinates for recordings in (substantia nigra pars compacta) SNc/VTA were within the following range: −3.8 to −2.9 mm from bregma, 4.0–4.8 mm from the surface, and 0.0–2.0 mm from the midline.

Immunohistochemistry and confocal microscopy

Transgenic mice expressing α6* nAChR subunits fused in-frame with green fluorescent protein (GFP) (α6GFP mice; 2 male/2 female) were anesthetized with sodium pentobarbital (100 mg/kg; i.p.) and transcardially perfused with 15 ml of ice-cold phosphate-buffered saline (PBS) followed by 25 ml of ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed for 2 h at 4°C. Coronal sections (50 μm) were cut on a microslicer and collected into PBS. Sections were permeabilized (20 mM HEPES, pH 7.4, 0.5% Triton X-100, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose) for 1 h at 4°C, blocked [0.1% Triton X-100, 5% donkey serum in Tris-buffered saline (TBS)] for 1 h at room temperature and incubated overnight at 4°C in solutions containing primary antibodies (diluted in 0.1% Triton X-100, 5% donkey serum in TBS). Primary antibodies and final dilutions were as follows: 1:500 rabbit anti-GFP (A11122; Invitrogen, Carlsbad, CA, USA), 1:500 sheep anti-tyrosine hydroxylase (AB1542, Millipore, Temecula, CA, USA) and 1:500 mouse anti-GAD67 (MAB5406; Millipore). Sections were washed three times for 10 min each in TBST (0.1% Triton X-100 in TBS) followed by incubation at room temperature for 1 h with secondary antibodies (diluted in 0.1% Triton X-100, 5% donkey serum in TBS). Secondary antibodies and final dilutions were as follows: 1:1000 goat anti-rabbit Alexa 488 (A11008; Invitrogen), 1:1000 donkey anti-sheep Alexa 555 (A21436; Invitrogen) and 1:1000 donkey anti-mouse Alexa 555 (A31570; Invitrogen). Sections were then washed four times in TBST for 10 min each. All sections were mounted on slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA), then imaged with a Nikon (Nikon Instruments, Melville, NY, USA) A1 laser-scanning confocal microscope system. Nikon Plan Apo × 10 air and × 60 oil objectives were used. Alexa 488 was excited with an argon laser at 488 nm, and Alexa 555 was excited with a yellow solid-state laser at 561 nm.

Patch clamp electrophysiology

Patch clamp electrophysiology was carried out as previously described (Engle *et al*. 2012) using tissue collected from adult mice (10 α6L9′S mice and 5 non-Tg mice). Each recorded cell within a slice was treated as a separate experiment, and each animal yielded between one and six recorded cells. Patch clamp experiments were not segregated by sex. A single slice was transferred to a 0.8 ml recording chamber (Warner Instruments, Hamden, CT, USA; RC-27L bath with PH-6D heated platform), and slices were superfused throughout the experiment with standard recording artificial cerebrospinal fluid (ACSF) (1.5–2.0 ml/min) containing (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 24 $NaHCO_3$, 12.5 glucose, 2 $MgSO_4{\cdot}7H_2O$ and 2 $CaCl_2{\cdot}2H_2O$ (300–310 mOsm, pH 7.3–7.4). Cells were visualized with an upright microscope (FN-1; Nikon) using infrared or visible differential interference contrast (DIC) optics. Patch electrodes were constructed from Kwik-Fil borosilicate glass capillary tubes (1B150F-4; World Precision Instruments, Inc., Sarasota, FL, USA) using a programmable microelectrode puller (P-97; Sutter Instrument Co., Novato, CA, USA). The electrodes had tip resistances of 4.5–8.0 MΩ when filled with internal pipette solution (pH adjusted to 7.25 with Tris base, osmolarity adjusted to 290 mOsm with sucrose) containing: 135 mM K^+ gluconate, 5 mM ethyleneglycoltetraacetic acid (EGTA), 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 2 mM Mg-ATP (adenosine triphosphate), and 0.1 mM guanosine triphosphate (GTP). Whole-cell recordings were taken at 32°C with an Axopatch 200B amplifier, a 16-bit Digidata 1440A A/D converter, and pCLAMP 10.3 software (all Molecular Devices; Sunnyvale, CA, USA). Data were sampled at 5 kHz and low-pass filtered at 1 kHz. The junction potential between the patch pipette and the bath solution was nulled immediately prior to gigaseal formation. Series resistance was uncompensated.

Ventral tegmental area DAergic neurons were identified as previously described (Drenan *et al*. 2008). These neurons typically exhibit broad spikes (>2 milliseconds), slow tonic firing (1–6 Hz) in coronal slice preparation, expression of significant I_h currents at hyperpolarized potentials, and significant sag responses in current clamp mode following injection of hyperpolarizing current. To examine the function of somatic nAChRs, ACh was locally applied using a Picospritzer III (General Valve, Fairfield, NJ, USA). Using a high-resolution micromanipulator (Sutter Instruments, Novato, CA, USA), the pipette tip was moved within 20–40 μm of the recorded cell 1–2 seconds before drug application. Acetylcholine-containing ACSF recording solution was then puffed at 10–20 psi for 250 milliseconds. Immediately after the application, the glass pipette was retracted.

Statistical analyses

Behaviours were analysed using analysis of variance (ANOVA) with the significance level set at $P \leq 0.05$. Between-group factors included genotype and sex. Within-group factors included concentration phase (3%, 6%, 10% and 20%), day, floor type (Grid or Hole), conditioning session (1–8), trial (4: one CS+ conditioning session and one CS− conditioning session), trial type (CS+ and CS−), block (2-day drinking averages), test (pretest and post-test), time bin (first and last 30 min) or min, where applicable. Only significant interactions including genotype are reported and followed-up with lower-order ANOVAs and *t*-tests, where applicable. Preference test data were averaged into 30 min time bins before analysis since treatment effects on the expression of CPP can change over the course of the 60-min preference test (e.g. Chester *et al*. 1998; Powers *et al*. 2010). Pearson correlations between CS+ time and locomotor activity during the post-test were conducted because locomotor activity can affect the expression of CPP (Gremel & Cunningham 2007).

Concentration–response curves for ACh-elicited inward currents in VTA neurons were derived by fitting mean peak current values for each ACh concentration to the Hill equation.

Results

Twenty-four-hour continuous-access

Alcohol intake

Similar to Kamens *et al*. (2010), mice were given access to escalating concentrations of alcohol over a 16-day test period in a two-bottle choice paradigm. Data from non-Tg mice (one male and one female) were excluded from analysis because of restricted fluid flow from the sipper tube, leaving a total of 33 mice included in analyses: 17 α6L9′S (9 male/8 female) and 16 non-Tg (9 male/7 female). Alcohol intake (g/kg) was averaged into 2-day blocks to simplify data presentation (Fig. 1a). The ANOVA [genotype (2) × sex (2) × concentration phase (4) × block (2: within each concentration)] indicated that females drank more than males ($F_{1,29} = 23.0$, $P < 0.01$) and that intake increased with increasing alcohol concentrations ($F_{3,87} = 34.9$, $P < 0.001$). A significant four-way interaction ($F_{3,87} = 4.7$, $P < 0.01$) was also found, and three-way ANOVAs (genotype × sex × block) within each concentration yielded significant three-way interactions at both the 3% ($F_{1,29} = 4.2$, $P = 0.001$) and 6% ($F_{1,29} = 7.9$, $P = 0.01$) concentrations, but not at 10% or 20%. Follow-up ANOVAs (genotype × sex) at each of the two blocks within the 3% and 6% concentrations yielded a main effect of genotype for block 1 (3% concentration; $F_{1,29} = 5.1$, $P < 0.01$; α6L9′S < non-Tg) and genotype × sex interactions for blocks 2 (3% concentration; $F_{1,29} = 5.4$, $P < 0.05$) and 4 (6% concentration; $F_{1,29} = 7.0$, $P = 0.01$). The interactions at blocks 2 and 4 resulted from female α6L9′S mice consuming more alcohol than female non-Tg mice ($Fs_{1,13} > 11.5$, $Ps < 0.01$).

Water intake

Water intake (ml/kg) was averaged into 2-day blocks, as was done for alcohol. The ANOVA [genotype (2) × sex (2) × concentration phase (4) × block (2: within each concentration)] uncovered a significant genotype × concentration phase × block interaction ($F_{3,87} = 9.8$, $P < 0.001$). Follow-up two-way ANOVAs (genotype × block) within each concentration phase indicated significant genotype × block interactions for the 3% and 6% concentrations ($Fs_{1,31} > 8.2$, $Ps < 0.01$). One-way ANOVAs comparing genotype at each block within the 3% and 6% concentration phases indicated that non-Tg drank significantly more water than α6L9′S mice on blocks 2 (3% concentration; $F_{1,29} = 17.7$, $P < 0.001$) and 4 (6% concentration; $F_{1,29} = 19.1$, $P < 0.001$), mirroring alcohol intake (data not shown).

Total fluid intake

Total fluid intake (ml/kg: Fig. 1b) was averaged into 2-day blocks, as was done for alcohol and water. The ANOVA [genotype (2) × sex (2) × concentration phase (4) × block (2: within each concentration)] indicated that females drank more total fluid than males ($F_{1,29} = 52.3$, $P < 0.001$). A significant four-way interaction ($F_{3,87} = 2.7$, $P < 0.05$) was followed-up with three-way ANOVAs within each concentration phase, but these analyses showed no significant effects.

Drinking in the dark

Mice removed from the data set in the 24-h drinking study (blocked tubes) were included in the DID study, for a total of 35 mice: 17 α6L9′S (9 male/8 female) and 18 non-Tg (10 male/8 female). A three-way ANOVA [genotype (2) × sex (2) × day (4)] of alcohol intake (g/kg) yielded a main effect of genotype ($F_{1,31} = 4.8$, $P < 0.05$), indicating that overall alcohol intake was greater in α6L9′S mice than non-Tg littermates (Fig. 2).

Alcohol-induced CPP

0.5 g/kg

Pretest preference. Analysis of the raw time spent on the Grid and Hole floors [genotype (2) × sex (2) × floor type (2) × time bin (2: first and last 30 min) ANOVA] yielded no effects indicating that mice did not show an unconditioned preference for either floor type.

Pretest activity. Analysis of pretest locomotor activity [genotype (2) × sex (2) × time bin (2: first and last 30 min) ANOVA] indicated that non-Tg mice were more active than α6L9′S mice ($F_{1,28} = 4.4$, $P < 0.05$) that females were more active than males ($F_{1,28} = 4.3$, $P < 0.05$), and that activity was lower during the last 30 min compared to the first 30 min ($F_{1,28} = 115.8$; $P < 0.01$) (data not shown).

Conditioning activity. Data from one female α6L9′S mouse was removed from conditioning activity analysis because of equipment malfunction on the second CS+ conditioning trial. Analysis of locomotor activity on CS+ and CS− trials [genotype (2) × sex (2) × trial (4) × trial type (2) ANOVA] indicated a main effect of sex ($F_{1,27} = 6.4$, $P < 0.05$; females > males) and a genotype × trial interaction ($F_{3,81} = 4.0$, $P = 0.01$). Follow-up analyses of genotype within each trial did not show significance; the interaction was due to a genotype dependent difference in reduced activity across trials.

A genotype × sex × trial type interaction ($F_{1,27} = 9.6$, $P < 0.01$) was also found. Follow-up analyses comparing genotype and sex within each trial type showed females were more active than males on both trial types ($Fs_{1,27} > 5.7$, $Ps < 0.05$). Comparing sex and trial type within each genotype indicated that α6L9′S females were more active than α6L9′S males ($F_{1,14} = 4.4$, $P = 0.05$), and non-Tg mice displayed greater activity on CS+ than on CS− trials ($F_{1,13} = 4.9$, $P = 0.05$) (Fig. 3); α6L9′S mice did not show a difference in activity levels between CS+ and CS− trial types.

Post-test preference. The ANOVAs comparing change in time spent on the CS+ floor from the pretest to the post-test [genotype (2) × sex (2) × test (2) × time bin (2: first and last 30 min)] indicated a significant genotype × sex × test interaction

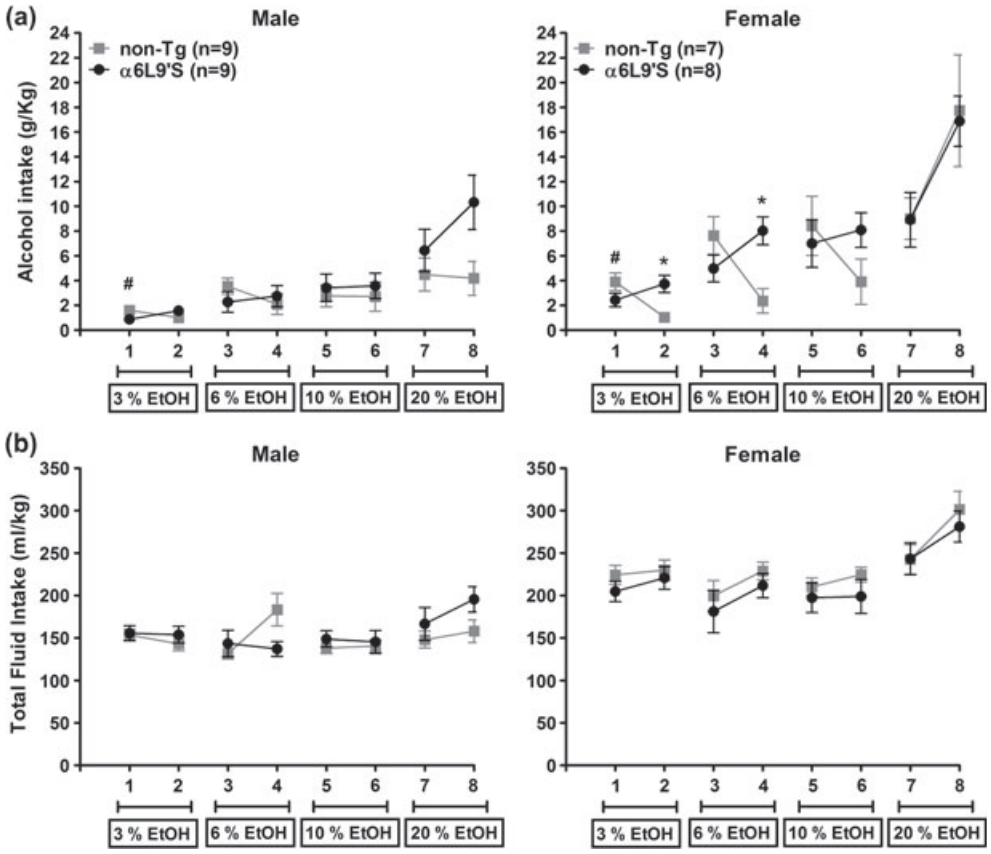

Figure 1: Data are shown as two-day averages (±SEM) of 24-h two-bottle choice (a) alcohol (g/kg) and (b) total fluid (ml/kg) intake for male (left panels) and female (right panels) α6L9′S and non-Tg mice. Female α6L9′S mice drank greater amounts of 3% and 6% alcohol solutions. No differences in total fluid intake were observed. *Indicates a significant difference of genotype ($P < 0.05$); #indicates a significant difference of genotype collapsed across sex ($P < 0.01$).

($F_{1,28} = 6.4$, $P < 0.05$), but follow-up analyses did not reach significance. There was also a genotype × test × time bin interaction ($F_{1,28} = 4.9$, $P < 0.05$). Two-way ANOVAs (genotype × test) within each time bin indicated a significant two-way interaction in the last 30 min only ($F_{1,28} = 6.1$, $P < 0.05$). Analysis of test within each genotype indicated a significant CPP in α6L9′S mice ($F_{1,16} = 4.4$, $P = 0.05$), but not in non-Tg mice (Fig. 4).

Post-test activity. Analysis of post-test locomotor activity [genotype (2) × sex (2) × time bin (2: first and last 30 min) ANOVA] indicated a genotype × time bin interaction ($F_{1,28} = 11.8$, $P < 0.01$). Genotype comparisons within each time bin indicated that non-Tg mice were more active than α6L9′S mice during the last 30 min only ($F_{1,30} = 13.6$, $P < 0.01$); there was a trend for the same genotype difference during the first 30 min ($P = 0.06$) (data not shown).

Pearson correlations. Correlations of CS+ time and post-test activity during the first and last 30 min indicated no significant relationship. The same analyses run within each genotype also indicated no significant relationships.

2.0 g/kg

Pretest preference. Analysis of the raw time spent on the Grid and Hole floors [genotype (2) × sex (2) × floor type (2) × time bin (2: first and last 30 min) ANOVA] yielded a genotype × sex × time bin interaction ($F_{1,24} = 4.7$, $P < 0.05$), but no floor type interaction, indicating that mice did not show an unconditioned preference for either floor type.

Pretest activity. Analysis of pretest locomotor activity [genotype (2) × sex (2) × time bin (2: first and last 30 min) ANOVA] indicated a significant sex × time bin interaction ($F_{1,24} = 8.4$, $P < 0.01$), due to a greater reduction in locomotor activity in males than females across the test session (data not shown).

Conditioning activity. Analysis of locomotor activity on CS+ and CS− trials [genotype (2) × sex (2) × trial (4) × trial type (2) ANOVA] indicated a significant trial × trial type interaction ($F_{3,72} = 9.7$, $P < 0.01$). Comparing trial within each trial type indicated a significant reduction in activity across CS− trials only ($F_{3,81} = 20.5$, $P < 0.01$). Comparing trial type within each trial indicated mice were significantly more active

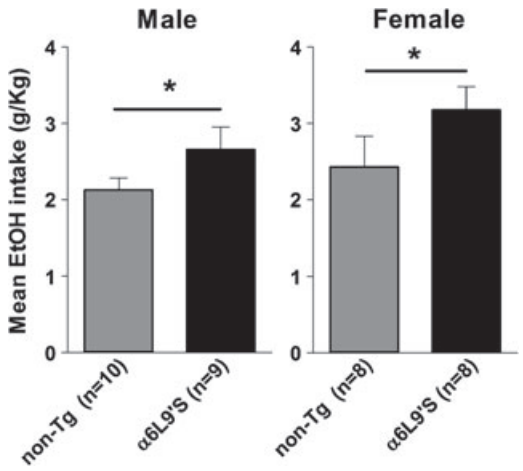

Figure 2: Data are shown as mean (±SEM) alcohol intake (g/kg) collapsed across the 4 days for male and female α6L9′S and non-Tg mice. α6L9′S mice showed greater binge-like alcohol drinking (DID) than non-Tg mice. *Indicates a significant main effect of genotype ($P < 0.05$).

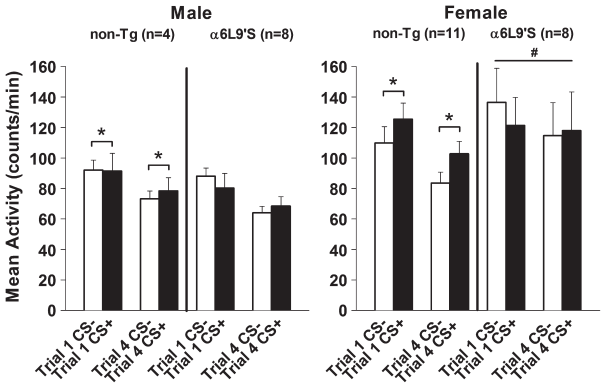

Figure 3: Data are shown as mean (±SEM) activity counts for α6L9′S and non-Tg mice during CS+ and CS− trials using a 0.5 g/kg conditioning dose of alcohol. Only activity from trials 1 and 4 are shown to simplify data presentation; trials 2 and 3 showed a similar pattern of activity differences as trial 4. Female α6L9′S mice were more active than male α6L9′S mice and non-Tg mice showed significantly greater activity on CS+ trials than CS− trials. *Indicates a main effect of trial type ($P < 0.05$; CS+ vs. CS−); # indicates significantly different from male α6L9′S mice ($P < 0.01$).

on CS+ days than CS− days for trials 2–4 [$Fs_{1,27} > 20.1$, $Ps < 0.01$ (Fig. 5)].

Post-test preference. The ANOVAs comparing change in time spent on the CS+ floor from the pretest to the post-test [genotype (2) × sex (2) × test (2) × time bin (2: first and last 30 min)] indicated a main effect of sex ($F_{1,24} = 6.3$, $P < 0.05$; male > female) and a strong trend for a main effect of test ($F_{1,24} = 4.0$, $P = 0.057$; post-test > pretest) (Fig. 6).

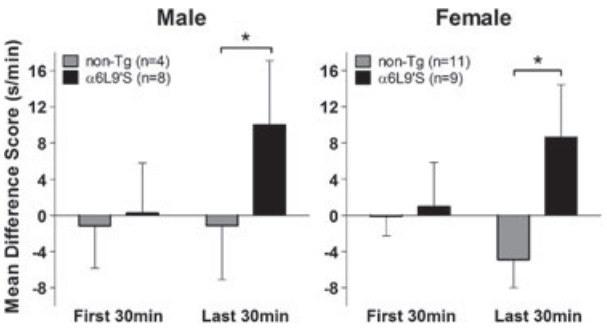

Figure 4: Data are shown as mean (±SEM) change in time (seconds/min) spent on the alcohol-paired floor between post-test and pretest (post-test – pretest difference score) for α6L9′S and non-Tg mice, during the first and last 30 min of the preference tests. Note that a bar above the 0 line indicates a preference for the alcohol paired cue, whereas a bar below the 0 line is indicates an aversion. Both male and female α6L9′S mice displayed a significant increase in time spent on the alcohol-paired floor as a result of conditioning with a 0.5 g/kg dose of alcohol, indicating that α6L9′S mice are more sensitive to the rewarding effects of alcohol. *Indicates main effect of genotype ($P < 0.05$).

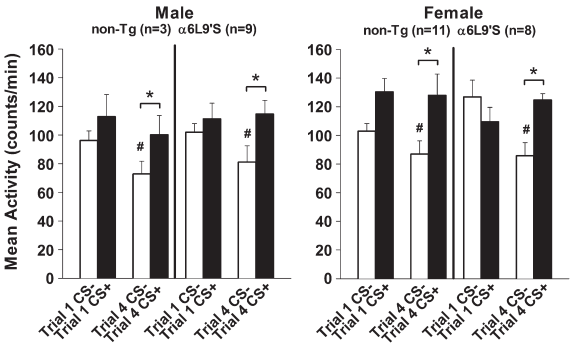

Figure 5: Data are shown as mean (±SEM) activity counts for α6L9′S and non-Tg mice during CS+ and CS− trials using a 2.0 g/kg dose of alcohol. All mice showed increased activity on CS+ trials 2–4 compared to CS− trials 2–4. All mice also showed habituation of activity across CS− trials. Only activity from trials 1 and 4 are shown to simplify data presentation. *Indicates a main effect of trial type ($P < 0.01$; CS+ vs. CS−); #indicates significantly different from Trial 1 CS− ($P < 0.01$).

Post-test activity. Analysis of post-test locomotor activity [genotype (2) × sex (2) × time bin (2: first and last 30 min) ANOVA] uncovered a main effect of time bin ($F_{1,24} = 33.6$, $P < 0.01$), indicating lower activity during the last than the first 30 min of the post-test (data not shown).

Pearson correlations. Correlations of CS+ time and post-test activity during the first and last 30 min indicated a significant negative relationship during the first 30 min ($r = -0.39$, $n = 28$, $P < 0.05$). The same analyses run within each genotype indicated significant negative relationships

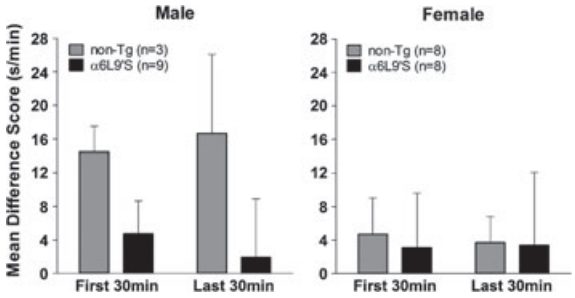

Figure 6: Data are shown as mean (±SEM) change in time (seconds/min) spent on the alcohol-paired floor between post-test and pretest (post-test – pretest difference score) for α6L9′S and non-Tg mice, during the first and last 30 min of the preference tests. Note that a bar above the 0 line indicates a preference for the alcohol paired cue. Analysis indicated a main effect of test ($P = 0.057$) very close to significant.

for non-Tg mice only, within both 30 min periods ($rs = -0.75$, $ns = 11$, $Ps = 0.01$).

Immunohistochemistry and confocal microscopy

To support the claim that α6* nAChRs are selectively expressed in DA neurons but not local GABA neurons, we studied α6GFP transgenic mice. α6GFP mice faithfully recapitulate the known, endogenous expression pattern of α6* nAChR subunits in the mouse brain (Mackey *et al*. 2012). We stained ventral midbrain sections in α6GFP mice with anti-GFP antibodies to localize α6* nAChRs, and we co-stained these sections for either tyrosine hydroxylase (TH; a marker of DA neurons) or glutamic acid decarboxylase 67 (GAD-67; a marker of GABAergic neurons).

Confocal microscopy showed the selective expression of α6* nAChRs (Fig. 7). Analysis of stained sections showed that while α6* nAChRs are indeed enriched in DA neurons in VTA and the adjacent substantia nigra pars compacta (SNc) (Fig. 7a), GABAergic neurons in ventral midbrain do not express α6* nAChRs (Fig. 7b). High-resolution confocal images at the border between SNc and SNr show that GFP-tagged α6 nAChR subunits are not localized in GAD67-positive, GABAergic neurons (Fig. 7c). Both male and female brains were analysed. We found no sex differences and no evidence for mis-expression of α6GFP subunits in brain areas not known to express this subunit (data not shown).

Patch clamp electrophysiology

α6* nAChRs in α6L9′S VTA DA neurons may enhance reward signals by being more strongly activated by endogenous ACh levels. To test this idea, we recorded from VTA DA neurons in acutely prepared brain slices from α6L9′S and control littermate mice. Following visual identification and the establishment of a stable whole-cell recording, VTA DA neurons were held in voltage clamp mode. A second, drug-filled micropipette was used to locally deliver ACh to the recorded neuron, resulting in activation of nAChRs (Fig. 8a; inset schematic). Acetylcholine-evoked currents were generated in response to lower concentrations of ACh in α6L9′S neurons relative to neurons from control littermate mice (Fig. 8a). Peak current amplitudes at each of several ACh concentrations were plotted, and the data were fitted to the Hill equation. In VTA DA neurons, the EC_{50} for ACh was lowered substantially in L9′S neurons compared to responses in control littermate neurons (non-Tg: 208 μM; α6L9′S: 2 μM; Fig. 8b). A similar EC_{50} trend was obtained from SNc DA neurons (non-Tg: 94 μM; α6L9′S: 1 μM; Fig. 8c). Together, these results show that nAChRs in α6L9′S VTA DA neurons are hypersensitive to ACh. This may partially underlie the increased sensitivity to alcohol reward in α6L9′S mice.

Discussion

Previous studies have utilized nAChR subunit knockout mice or pharmacological blockade of nAChRs to investigate the relevant nAChR subunits involved in alcohol reward. Here, we studied α6L9′S mice, which were specifically engineered to amplify and/or isolate functional responses following α6* nAChR activation. α6GFP mice were also used to precisely localize α6* nAChRs in individual neurons in the mesolimbic reward system. We found that female α6L9′S mice drank greater amounts of 3% and 6% alcohol solutions in a two-bottle choice paradigm. α6L9′S of both sexes drank greater amounts of a 20% alcohol solution in the DID paradigm. Further, α6L9′S mice exhibited CPP following injection of 0.5 g/kg alcohol, which was insufficient to produce CPP in littermate control mice. Both genotypes showed evidence of CPP in the 2.0 g/kg experiment, although the main effect did not quite reach statistical significance ($P = 0.057$). Our data also showed that α6* nAChRs are specifically expressed in midbrain DAergic (but not GABAergic) neurons (Fig. 7), and that α6* nAChRs in α6L9′S mice are dramatically more sensitive to endogenous ACh (Fig. 8), suggesting that DA reward signals following alcohol exposure are enhanced in α6L9′S mice. Taken together, these results suggest that genotype, sex and alcohol concentration are interacting to influence sensitivity to the rewarding effects of alcohol.

α6 nAChRs in alcohol reward-related behaviours*

Interest in studying the modulation of alcohol's rewarding effects by the nicotinic cholinergic system stems from the high co-morbidity between tobacco addiction and alcohol use disorders (AUDs) (Dani & Harris 2005). Nicotine and/or alcohol interactions at specific nAChR subtypes may explain the co-morbidity. Indeed, particular alleles in either of the α5-α3-β4 or α6-β3 nAChR gene clusters have been associated with increased risk toward development of nicotine addiction and AUDs in humans (Hoft *et al*. 2009; Wang *et al*. 2009), though α5* nAChRs do not play a role in alcohol consumption in mice (Santos *et al*., 2012).

Enhanced alcohol consumption in female α6L9′S mice in the 24-h drinking experiment and in both male and female α6L9′S mice for the DID experiment is consistent with prior studies in which nAChR subtypes have been implicated in alcohol intake and alcohol reward. Mecamylamine, a non-specific nAChR antagonist, reduced voluntary alcohol intake using both 24-h and limited-access drinking procedures

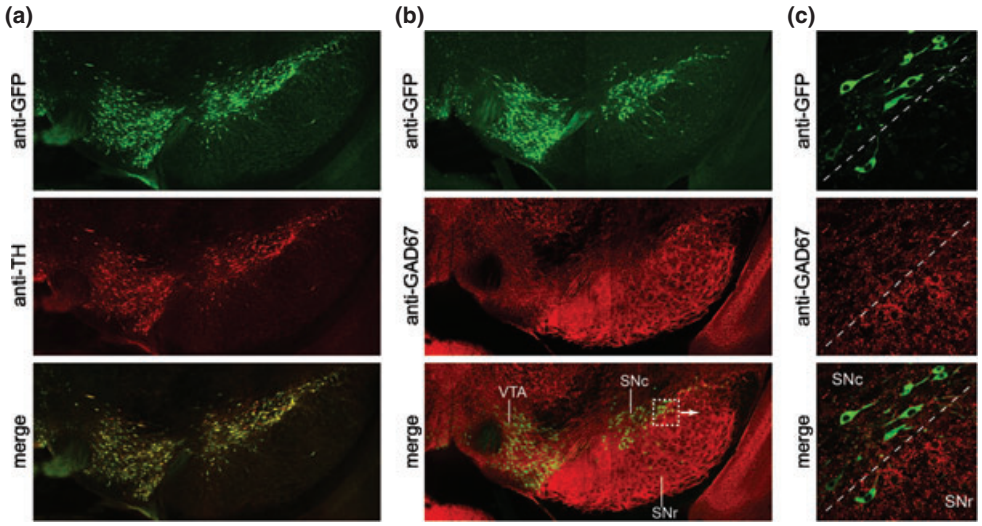

Figure 7: α6* nAChRs are selectively expressed in midbrain DA neurons. Ventral midbrain sections from transgenic α6GFP mice were stained with anti-GFP and anti-TH (a) or anti-GFP and anti-GAD67 (b) antibodies and imaged using confocal microscopy. High resolution scans at the border between the DA neuron-rich SNc and the GABA neuron-rich SNr (b, white box) were made, showing that α6* nAChRs are excluded from GABA neurons (c). (The colour version of Fig. 7 may be found in the online version of this article at the publisher's web-site.)

(Blomqvist *et al*. 1996; Le *et al*. 2000), and reduced alcohol intake in a binge-drinking paradigm (Hendrickson *et al*. 2009). Varenicline, an FDA-approved smoking cessation drug and partial agonist at $\alpha4\beta2$* and $\alpha6\beta2$* nAChRs (Bordia *et al*. 2012; Mihalak *et al*. 2006) acts via VTA nAChRs to attenuate alcohol consumption (Hendrickson *et al*. 2010; Zhao-Shea *et al*. 2011). Mice lacking α4 nAChR subunits – a genetic alteration that dramatically reduces α6* nAChR function (Drenan *et al*. 2010) – show significantly less alcohol consumption than mice with intact α4* nAChR function (Hendrickson *et al*. 2010). Although mice lacking α6 nAChR subunits (α6KO mice) showed increased sensitivity to the sedative-hypnotic effects of high alcohol concentrations, they did not show differences in alcohol consumption (Kamens *et al*. 2012). These results could be due to $\alpha4\beta2$* nAChR up-regulation, which is seen in α6KO DA terminals (Champtiaux *et al*. 2003). Importantly, blockade of α6* nAChRs in the VTA with α-conotoxin MII reduced locomotor activity, accumbal DA release, and the rewarding action of alcohol (Larsson *et al*. 2004). Local VTA infusion of α-conotoxin MII also blocked alcohol-associated conditioned responding (Larsson *et al*. 2004). These pharmacological and/or genetic loss-of-function studies of α4* and α6* nAChRs complement the data we present here; prior results in mice with diminished function at α4* or α6* nAChRs showed reduced alcohol consumption or reward, and in this article, enhanced α6* nAChR function is associated with enhanced sensitivity to the rewarding effects of alcohol.

Intriguingly, we found a sex-dependent genotype difference in alcohol intake at the 3% and 6% concentrations, such that female α6L9′S mice drank significantly more than female non-Tg mice (Fig. 1). These differences in alcohol intake are not related to polydipsia since both genotypes consumed similar amounts of total fluid, which implies the observed differences are specific to alcohol. Future studies comparing intake of other tastants (e.g. saccharine/quinine preference) will be important and allow for assessment of reward-related genotype differences with other reinforcers. These results suggest a leftward shift in the alcohol reward dose response curve in female α6L9′S mice. It is interesting to note that while sex-dependent genotype differences were uncovered in the 24-h drinking paradigm, there were no significant sex-dependent effects in DID or the expression of CPP. α6L9′S mice, due to the discrete and well-described nature of their genetic alteration, will be useful in future studies to model sex differences in alcohol and/or nicotine reinforcement.

The increased sensitivity to alcohol-induced CPP at the 0.5 g/kg dose in α6L9′S mice provides further support for the idea that the L9′S mutation produced a leftward shift in the dose–response curve for alcohol reward in these mice (Fig. 4). Similarly, Liu and colleagues (2012a) recently reported CPP with a 0.5 g/kg alcohol dose in mice with hypersensitive α4* nAChRs (Leu9′Ala) but not in wildtype mice; no genotype difference was observed with 2.0 g/kg alcohol-induced CPP. Interestingly, α6 subunits are co-expressed with α4 subunits in VTA DAergic neurons (Champtiaux *et al*. 2003; Liu *et al*. 2012b), suggesting that α6* and α4*, or possibly $\alpha6\alpha4$* nAChRs, are involved in modulating alcohol reward-related behaviour.

Although we observed no genotype differences in the locomotor-activating effects of alcohol during the CS+ conditioning trials, genotype differences were observed in the absence of alcohol. Non-Tg mice were more active than α6L9′S mice during both the pretest and post-test of the 0.5 g/kg alcohol study, but not the 2.0 g/kg study. This result is inconsistent with a previous report indicating greater

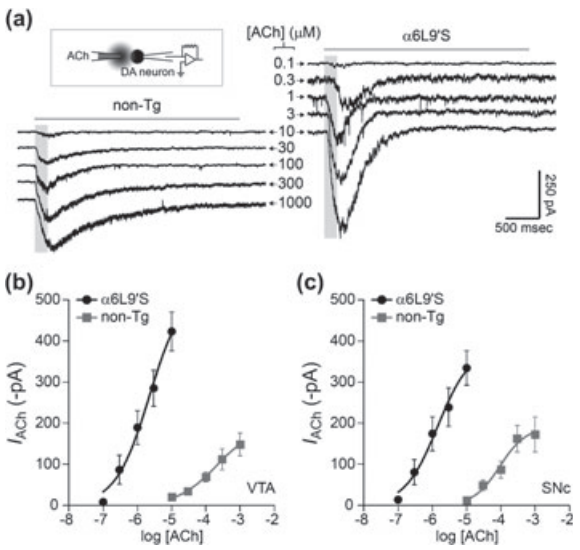

Figure 8: Nicotinic acetylcholine receptors in midbrain DA neurons of α6L9′S mice are hypersensitive to ACh. Ventral tegmental area DA neurons in acutely prepared brain slices from α6L9′S or non-Tg littermate mice were studied with whole-cell patch clamp recordings. A range of ACh concentrations were directly applied to the recorded DA neuron using a puffer pipette (a, inset), resulting in nAChR-mediated inward currents in α6L9′S and non-Tg cells (a). Concentration-response curves for ACh-activated inward currents in VTA (b) and SNc (c) DA neurons were generated from the experimental data described in (a).

home-cage activity in α6L9′S than non-Tg mice (Cohen *et al*. 2012; Drenan *et al*. 2010, 2008), but it could be explained by environmental or procedural factors that differ between this and the prior study. It should be noted that the genetic background of the α6L9′S mice in both studies was C57BL/6.

Correlations between CS+ time and activity during the post-test yielded a significant negative relationship in non-Tg but not in α6L9′S mice in the 2.0 g/kg CPP experiment; no correlation was found in the 0.5 g/kg alcohol experiment. Gremel and Cunningham (2007) have previously shown a negative relationship between CPP magnitude and test activity, suggesting that higher levels of activity interfere with the expression of CPP (Gremel & Cunningham 2007). This negative relationship found in the non-Tg mice (and lack thereof in the α6L9′S mice) may explain the weaker 2.0 g/kg alcohol-induced CPP in the α6L9′S mice (Fig. 6).

α6 nAChR neurobiology and the response to alcohol*

Studies of the behavioural and neurobiological action of α6* nAChRs are of intense interest for two key reasons: (1) α6* nAChRs respond robustly to concentrations of nicotine that are well within the physiological range of nicotine concentrations found in the cerebrospinal fluid of smokers (Salminen *et al*. 2007), implicating their role in nicotine addiction and as targets for smoking cessation drugs (Quik & Mcintosh 2006) and (2) α6* nAChRs are selectively expressed in only a few brain regions (Azam *et al*. 2002; Champtiaux *et al*. 2002; Drenan *et al*. 2008; Le Novere *et al*. 1996), the most important being the mesolimbic and nigrostriatal DA systems. Selective manipulation of nAChR activity in these neurotransmitter systems by α6* nAChR agonists, antagonists, or positive allosteric modulators could provide new avenues for development of treatments for Parkinson's disease, smoking cessation, and AUDs (Drenan & Lester 2012).

Alcohol's action in the brain is complex, likely involving a variety of interactions at specific neurotransmitter receptors, ion channels, and brain circuits in a concentration-dependent manner (Morikawa & Morrisett 2010). Ventral tegmental area DA neuron activity, specifically, baseline spontaneous firing rate, and the transition from tonic to phasic firing are key neural correlates of alcohol reward (Pfeffer & Samson 1988; Rassnick *et al*. 1992). Cholinergic excitation of nAChRs is permissive for activation of DA neuron phasic firing (Mameli-Engvall *et al*. 2006) and for self administration of several drugs of abuse (Corrigall *et al*. 1994). Alcohol has well-known effects on $GABA_A$ receptors (Whitten *et al*. 1996) and NMDA receptors (Lovinger *et al*. 1989; Maldve *et al*. 2002) that likely play a role in alcohol addiction. Importantly, several studies support the idea that alcohol may directly potentiate nAChR activity in VTA DA neurons. Co-application of alcohol was sufficient to potentiate peak current amplitude of β4* nAChRs (Borghese *et al*. 2002; Covernton & Connolly 1997), α-bungarotoxin insensitive nAChRs in primary cortical neurons (Aistrup *et al*. 1999; Marszalec *et al*. 1999) and α4β2 nAChRs (Zuo *et al*. 2001, 2002). On the basis of these studies and the results we present in this article, alcohol may also potentiate α6* nAChR responses. Future studies will be required to address this hypothesis.

In patch-clamp recordings from VTA DA neurons in acute brain slices, activation of nAChRs with low concentrations of nicotine potentiated an alcohol-induced increase in action potential firing (Clark & Little 2004). Furthermore, Taylor and colleagues showed that infusion of nicotine into VTA was sufficient to summate with low-concentrations of alcohol to enhance DA release in nucleus accumbens (Tizabi *et al*. 2002). These results suggest that low-concentrations of alcohol or low-levels of nAChR activity, which alone may not be sufficient to support changes in behaviour, may combine to produce sufficient DA neuron activity to produce the rewarding effects of alcohol. In α6L9′S mice, stronger levels of ACh-activated α6* nAChR activity (Fig. 8) may combine with alcohol concentrations that are normally subthreshold (3–6% solutions: Fig. 1; 0.5 g/kg alcohol: Fig. 4) to produce sufficient stimulation of the mesolimbic DA system to produce alcohol reward. This supposition is strongly supported by recent VTA DA neuron patch clamp experiments with hypersensitive α4* nAChR mice, where alcohol was able to summate with bath-applied ACh to increase firing rates specifically via α4* nAChRs (Liu *et al*. 2012a).

Conclusions

This study is the first to directly show a role for α6* nAChRs in modulating alcohol reward-related behaviours. The data suggest that α6L9′S mice are more sensitive to

alcohol reward, likely a result of hypersensitive α6* nAChRs. These results using gain-of-function nAChRs expressed in mice, together with the emerging literature detailing the roles of α6* nAChRs in DA neurobiology (Drenan *et al*. 2010, 2008; Mackey *et al*. 2012), suggest that α6* nAChRs are a promising therapeutic target for the treatment of AUDs.

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